



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : A23K 3/03, C12N 9/24 A23K 1/165		A1 (11) International Publication Number: WO 92/01389 (43) International Publication Date: 6 February 1992 (06.02.92)
(21) International Application Number: PCT/NL91/00136 (22) International Filing Date: 24 July 1991 (24.07.91)		(74) Agents: HUYGENS, Arthur, Victor et al.; Gist-Brocades N.V., Patents and Trademarks Dept., Wateringseweg 1, P.O. Box 1, NL-2600 MA Delft (NL).
(30) Priority data: 90202020.5 24 July 1990 (24.07.90) (34) Countries for which the regional or international application was filed:		EP (81) Designated States: AU, BG, CA, FI, HU, JP, KR, NO, + RO, SU, US. Published <i>With international search report.</i>
(71) Applicant (for all designated States except US): GIST-BROCADES N.V. [NL/NL]; Wateringseweg 1, P.O. Box 1, NL-2600 MA Delft (NL).		
(72) Inventor; and (75) Inventor/Applicant (for US only) : HARDER, Abraham [NL/NL]; De Vaert 9, NL-2651 EP Berkel en Rodenrijs (NL).		
(54) Title: ENZYMATIC TREATMENT OF SILAGE		
(57) Abstract <p>The present invention provides a silage composition supplemented with endo-xylanase activity. According to the present invention, the addition of endo-xylanase to fodder produces a silage having a significant increase in the amount of water soluble carbohydrates for use by lactic acid bacteria or as nutrients for the animal ingesting the silage; and increased fermentative production of lactic acid and acetic acid by lactic acid bacteria and the consequent lowering of pH which serves to preserve the silage composition. Also provided are silage compositions which further contain an inoculum of a lactic acid bacteria.</p>		

+ DESIGNATIONS OF "SU"

It is not yet known for which States of the former Soviet Union any designation of the Soviet Union has effect.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	ES	Spain	MG	Madagascar
AU	Australia	FI	Finland	ML	Mall
BB	Barbados	FR	France	MN	Mongolia
BE	Bulgium	GA	Gabon	MR	Mauritania
BF	Burkina Faso	GB	United Kingdom	MW	Malawi
BC	Bulgaria	CN	Guinea	NL	Netherlands
BJ	Benin	GR	Greece	NO	Norway
BR	Brazil	HU	Hungary	PL	Poland
CA	Canada	IT	Italy	RO	Romania
CP	Central African Republic	JP	Japan	SD	Sudan
CC	Congo	KP	Democratic People's Republic of Korea	SE	Sweden
CH	Switzerland	KR	Republic of Korea	SN	Senegal
CI	Côte d'Ivoire	L1	Liechtenstein	SU+	Soviet Union
CM	Cameroon	LK	Sri Lanka	TD	Chad
CS	Czechoslovakia	LU	Luxembourg	TG	Togo
DE	Germany	MC	Monaco	US	United States of America
DK	Denmark				

ENZYMATIC TREATMENT OF SILAGE

5

The present invention relates to the field of agriculture, more specifically, the present invention relates to an improvement in the enzymatic treatment of fodder silage.

10

Background of the Invention

Improvements in silage technology have, next to natural and artificial drying, made ensiling the most widely applied technology for the preservation of forage. It has 15 been estimated that in western Europe, 60% (approximately 77 million tons dry matter) of the forage preserved for winter is ensiled. In the U.S., approximately 80 million tons (dry matter) are ensiled per year. Ensiling is also widely used in eastern Europe and Canada.

20

However, effluent production and nutrient losses in silage caused by fermentation (especially butyric acid fermentation) and aerobic deterioration have become increasingly problematic with the growing demand for higher efficiency in animal livestock production. These problems 25 often depend on factors such as the type of crop, climactic conditions and available technology. Recently introduced production limits for dairy farming and, in some countries, environmental legislation indirectly restricting the type and amounts of nutrients used for livestock production 30 emphasize the need to feed high quality silages (Spoelstra, S.F. (1991) In: Proceedings of the EGF Conference - "Forage Conservation Towards 2000"; Braunschweig, Germany).

35

Grasses (such as rye-grasses, etc.), cereals (such as maize, sorghum, wheat, etc.) and legumes (such as alfalfa, clover, etc.) are the primary crops used as fodder for ensiling. Beet tops and sugar beet pulp are also used in relatively smaller amounts.

- 2 -

Maize ensiles well, but is susceptible to aerobic deterioration. Grass, mainly perennial rye-grass, is often heavily fertilized and has, as a result, a low ratio of water soluble carbohydrates to buffering capacity making it 5 susceptible, after ensiling, to butyric acid fermentation. Butyric acid fermentation leads to dry matter losses in the silage, thus lowering the nutritional value and consequently, livestock production efficiency.

To avoid undesirable butyric acid fermentation, the 10 grass is normally wilted in the field to, on average, 500 g DM (dry matter) per kg. Alternatively, at DM contents below 350 g DM per kg, additives such as enzymes are applied.

Continuing research efforts have been undertaken to 15 study whether silage preservation is enhanced and silage intake and digestibility is improved by the addition of cell wall degrading enzymes, cellulases and hemicellulases, in particular.

The actions of cellulases and hemicellulases liberate 20 water soluble carbohydrates from the structural polysaccharides of the plant cell wall, thus providing substrates for the production of lactic acid via fermentation by the lactic acid bacteria present in the silage. These enzymes are probably also responsible for the biolytic disruption of the plant cell walls, releasing 25 further water soluble carbohydrates and other materials from within the cell for use by the lactic acid bacteria. In addition, the pre-digestion of the plant cell walls during the storage of the silage may lead to their being more rapidly degraded in the rumen and thus enhance digestibility 30 and moreover may provide increased amounts of liberated carbohydrates for added nutritional value for the animal ingesting the thus-treated silage.

The production of lactic acid (as well as acetic acid) 35 by lactic acid bacteria present in the silage is also responsible for the lowering of the pH of the silage. The lowering of the pH, generally decreasing to about pH 4.5 and

- 3 -

5 may be as low as about pH 4, creates an unfavorable environment for the growth of yeasts, as well as many other undesired microbes such as butyric acid-producing microorganisms, thus preserving the silage and maintaining its nutritional content.

Additionally, the lactic acid bacteria also serve as probiotics, beneficially enhancing the intestinal flora of the animal ingesting the silage.

10 Most, if not all enzyme preparations investigated for use in silage are rather crude fermentation products from fungi. These enzyme preparations contain many different enzyme activities.

15 For example, a cellulase preparation, obtained from a Penicillium verruculosum mutant strain, is disclosed in East German patent publication DD-278359 (published May 2, 1990) to be applicable for partial or total hydrolysis of cellulose and hemicellulose in industrial processes such as the production of silage, *inter alia*. The cellulase preparation is purported to contain high cellulolytic 20 activity combined with beta-glucosidase, xylanase and amylase activity.

25 A probiotic inoculum for silage comprising lactic acid bacteria transformed with exogenous DNA encoding an enzyme capable of degrading polysaccharides and oligosaccharides in a silage crop to provide a source of water soluble carbohydrates for the bacteria themselves is described in PCT Application WO 89/01970 (published March 9, 1989).

Summary of the Invention

30 According to the present invention, it has been found that endo-xylanase activity, of the many enzymatic activities present in enzymatic preparations used to ensile fodder, is particularly responsible for the release of water soluble carbohydrates, which through the fermentative action 35 of lactic acid bacteria, leads to the preservation and improved nutritional quality of the silage. Furthermore, it

has been found that supplementing fodder to be ensiled with sufficient amounts of endo-xylanase, either with or without the presence of other enzymatic activities, provides optimal preservation and nutritional quality of the thus-treated silage. It has also been observed that animals ingest greater amounts of silage preserved in this manner, as opposed to silage which has deteriorated. These factors all lead to an enhancement in the efficiency of livestock production.

The present invention thus provides a silage composition supplemented with an amount of endo-xylanase activity effective in releasing water soluble carbohydrates from the ensiled plant cell material (fodder), thus enhancing the production of lactic acid and acetic acid by lactic acid bacteria, which in turn improves the preservation and nutritional quality of the silage. The endo-xylanase is preferably added in a form which is essentially free of other enzymatic activity.

As the fodder to be ensiled does not always contain sufficient amounts of lactic acid bacteria to achieve proper ensiling, the present invention also provides silage compositions which further contain an inoculum of a lactic acid bacteria, included as a supplement in an amount sufficient to assist the preservation of the silage and to act as probiotics for the enhancement of the intestinal flora of the animal ingesting the thus-treated silage composition.

It is also an object of the present invention to provide a method of preserving silage characterized in that the silage is supplemented with an amount of endo-xylanase activity effective in increasing the amount of water soluble carbohydrates present in the silage for use in the production of lactic acid and acetic acid by lactic acid bacteria.

It is a further object of the present invention to provide a method for the improvement of the nutritional

- 5 -

value of silage by supplementing the silage with an amount of endo-xylanase activity effective in releasing water soluble carbohydrates from the ensiled plant cell material for utilization by the animal ingesting the silage. The 5 endo-xylanase present in the silage, also provides a supplement of an essential enzyme for the animal itself.

It is yet another object of the present invention to provide a method for improving the in vivo (dry matter) digestibility of silage in animals wherein the animal is fed 10 a diet consisting at least in part of a silage which has been supplemented with an amount of endo-xylanase activity effective in at least partially degrading, and thus pre-digesting, plant cell walls.

15 Brief Description of the Figure

Figure 1: HPLC elution profile of a culture filtrate obtained from Aspergillus niger DS16813 (CBS 323.90). This strain was later reclassified as 20 more likely belonging to the species Aspergillus tubigensis.

Detailed Description of the Invention

According to the present invention, fodder to be ensiled is supplemented with an amount of endo-xylanase 25 which is effective in producing a silage having a significant increase in the amount of water soluble carbohydrates for use by the lactic acid bacteria or as nutrients for the animal ingesting the silage; and improved qualities such as increased fermentative production of 30 lactic acid and acetic acid (lactic acid being produced in greater quantities relative to acetic acid) by (probiotic) lactic acid bacteria and the consequent lowering of pH which serves to preserve the silage composition and its nutritional value, these factors contributing to the 35 improvement of livestock production efficiency. Until the present invention, endo-xylanases were not known to be

specifically responsible for such a significant role in the production of improved silages. Nor was it known that the addition of an endo-xylanase in a form which is substantially free of other enzymatic activity (especially other cell wall degrading enzymatic activity) to fodder to be ensiled, would lead to obtaining a silage which is not only better preserved, but has improved nutritional quality as well.

Silage compositions, according to the present invention, preferably contain more than 50,000 units endo-xylanase activity per kg (fresh weight) fodder. The endo-xylanase optimally has a pH optimum in the range of pH 3.5 to pH 6.0 in order to ensure that maximum enzyme efficacy is obtained at the acidic pH conditions which exist in the process of ensiling fodder. As stated above, the endo-xylanase is preferably added in a form which is substantially free of other enzymatic activity in general, and cell wall degrading enzymatic activity in particular.

The supplementation of the fodder to be ensiled with endo-xylanase activity which is substantially free of other enzymatic activities is also commercially attractive as a more cost-effective alternative to the use of enzyme mixtures wherein secondary enzyme activities often must be supplemented to obtain the desired effect. The phrase "substantially free of other enzymes", as defined in context of the present invention, intends that the majority (i.e. at least 50% per weight, preferably at least 80% per weight) of the total protein found in the enzymatic preparation is endo-xylanase.

For example, the endo-xylanase activity may be produced in large quantities using recombinant DNA techniques, such as described in European Patent Application no. 90202020.5 (filed July 24, 1990), the disclosure of which is hereby incorporated by reference. Alternatively, the production of endo-xylanases may be enhanced by adjusting the fermentation conditions of the endo-xylanase-

producing microorganism such as the use of an inducing media containing xylan.

Endo-xylanases, for use in the context of the present invention, are preferably obtained from a filamentous fungus, more preferably from a filamentous fungus selected from the genera consisting of Aspergillus, Disporotrichum, Penicillium, Neurospora, Fusarium and Trichoderma, and most preferably obtained from a filamentous fungus selected from the species consisting of Aspergillus niger, Aspergillus awamori, Aspergillus aculeatus, Aspergillus tubigensis, Disporotrichum dimorphosporum and Trichoderma reesei.

An endo-xylanase to be used in the present invention may be identified via assay methods not critical to the present invention, such as a spot test assay. According to this method, a filtrate obtained from the culturing of a microorganism induced (e.g. with oat spelt xylan) to produce an endo-xylanase may be tested for the presence of endo-xylanase activity. Drops of the elution fractions are placed individually onto an agar film containing a citrate-phosphate buffer (see Example 1.1, below) and oat spelt xylan. The film is then incubated. If endo-xylanase activity is present, the locations of the individual drops on the agar film are visibly clear.

Endo-xylanase activity may also be identified by subjecting a xylan-containing solution to an enzyme solution suspected of containing endo-xylanase activity and spectrophotometrically analyzing the reducing sugars by the method as described by Leathers, T.D. *et al.* (1984) *Biotechnol. Bioeng. Symp.*, 14, 225.

A unit of endo-xylanase activity is herein defined as the amount (mol) of xylose equivalents liberated per minute per mg prc. in at a temperature of 39°C and at pH 3.5. Protein determination was performed according to the method as described by Bradford, M.M. (1976) *Anal. Biochem.*, 72, 248. Bovine immuno-gamma-globulin (B1gG) was used as the protein standard.

Once identified, an endo-xylanase-producing organism may be fermented under conditions conducive to the production of endo-xylanase and the desired enzymatic activity may be isolated from the culture filtrate of the 5 production organism and, if desired, purified by conventional means not critical to the present invention. For example, affinity and/or ion exchange chromatography techniques may advantageously be used for the preparative purification of an endo-xylanase from a culture filtrate.

10 Another embodiment of the present invention also envisions the supplementation of commercial enzyme preparations with endo-xylanase activity. Preferably, an amount of endo-xylanase is added to provide a total endo-xylanase activity in the final mixture of more than 50,000 15 units/kg fresh weight fodder.

15 In yet another embodiment of the present invention silage compositions are provided which further contain an inoculum of a lactic acid bacteria, included as a supplement to assist in the preservation of the silage. The lactic acid 20 bacteria is preferably selected from the group consisting of the genera Lactobacillus, Enterococcus, Lactococcus, Pediococcus and Leuconostoc. According to the present 25 invention, the most preferred lactic acid bacteria is selected from the group consisting of Lactobacillus plantarum, Lactobacillus acidophilus, Lactobacillus brevis, Lactobacillus bulgaricus, Lactobacillus fermentum and Lactobacillus lactis.

30 The supplementation of endo-xylanase to silage, according to the present invention may be applied to all ensilable plant material such as grasses, cereals and legumes, as known to those skilled in the art. The fodder normally, however, will have a dry matter (DM) content of below 350 g DM per kg fodder (fresh weight) to allow the 35 enzyme free access to the plant cell material.

35 The following examples are provided so as to give those of ordinary skill in the art a complete disclosure and

- 9 -

description of how to make and use the invention and are not intended to limit the scope of what the inventors regard as their invention. Efforts have been made to ensure accuracy with respect to numbers used (e.g., amounts, temperature, 5 pH, etc.) but some experimental errors and deviation should be accounted for. Unless indicated otherwise, temperature is in degrees Celsius and pressure is at or near atmospheric.

Example 1

10 Purification of Aspergillus tubigensis endo-xylanase. A culture filtrate was obtained by the culturing of Aspergillus niger DS16813 (CBS 323.90, deposited at the Centraal Bureau voor Schimmelcultures, Baarn, The Netherlands on July 20, 1990 - later reclassified as more 15 likely belonging to the species A. tubigensis; Kusters-van Someren et al. (1991) Curr. Genet. 19, 21) in a medium containing (per liter): 30 g oat spelt xylan (Sigma); 7.5 g NH₄NO₃, 0.5 g KCl, 0.5 g MgSO₄, 15 g KH₂PO₄, and 0.5 g yeast extract (pH 6.0). The culture filtrate was concentrated to a 20 volume of approximately 35 ml which was then was ultrafiltered on a Diaflo PM 10 filter in a 50 ml Amicon module to remove salts.

The supernatant was then concentrated to a volume of 10 ml and the retentate was washed twice with 25 ml 25 mM 25 Tris-HCl buffer (pH 7.0). After washing, the retentate volume was brought to 25 ml.

This retentate was injected in 1 ml quantities onto a Syn Chropak AX 300 column (dimensions 10 x 250 mm) and eluted in the following HPLC regime:

30

- 10 -

elution rate: 2 ml/min.
elution buffer A: 25 mM Tris-HCl pH 7.0
elution buffer B: 25 mM Tris-HCl pH 7.0 + 1 M NaCl
elution gradient: time

		<u>(min)</u>	<u>%A</u>	<u>%B</u>
5		0	99	1
		12	97	3
		30	80	20
		50	50	50
10		70	0	100
		90	0	100
		95	99	1

Fractions of 1 ml each were collected. Detection of the eluted protein was performed by continuous measurement of the UV absorption at 280 nm. The elution profile is shown in Figure 1.

The fractions were tested for the presence of endo-xylanase activity by a spot test. This spot test consists of adding 12 ml citrate-phosphate buffer (prepared by mixing 900 ml 0.2 M Na_2HPO_4 and 125 ml 0.5 M citric acid, followed by an adjustment of the pH of the solution to pH 5.6 using 0.5 M citric acid or 0.2 M Na_2HPO_4) containing 0.5% oat spelt xylan (Sigma) to 180 mg agar (Difco) and heating the mixture to 100°C to dissolve the agar. After cooling to 60°C, the agar mixture is poured evenly onto Agarose gel-bond film. Drops of the elution fractions are placed individually onto the film and incubated for 30 min. at 30°C. If endo-xylanase activity is present, the location of the individual drops on the agar film is clear.

Total xylanase activity in the collected fractions was quantitatively determined by measuring the amount of reducing sugars produced over a predetermined time period in the microassay as described by Leathers *et al.* (1984), using oat spelt xylan in 50 mM sodium acetate at pH 5.0 as a substrate. Activity units are also as defined by Leathers (*supra*).

Exo-xylanase activity in the eluted fractions was determined by the method described by Poutanen and Puls (1988), using p-nitro-phenyl- β -D-xylopyranoside (0.3 mM, Sigma) as a substrate at pH 5.0 and 30°C.

- 11 -

The spot test revealed that the elution fractions corresponding to peaks B, F and K (see Figure 1) contain endo-xylanase activity. The total xylanase assay showed activity in the elution fractions of peaks B, F, H and K.

5 The elution fractions of peaks B and H were determined to contain exo-xylanase activity.

The elution fractions of peaks F (XYL2 protein) and K (XYL A protein) were further purified by repeated ion exchange chromatography.

10

Example 2

Perennial English rye-grass was mown, dried for 24 hours until reaching a dry matter (DM) content of about 25%, cut into lengths of 1-2 cm and ensiled in 1 liter laboratory silos and stored for 3 months at ambient temperature before analysis.

The enzyme preparation Cellulase ABG-7 was added at a concentration of 0.2 g of protein per kg fresh weight grass (equivalent to 3200 units of endo-xylanase activity per kg fodder; protein content determined using the method as described by Bradford, M.M. (supra)). Cellulase ABG-7 is a commercial enzyme preparation produced by Trichoderma reesei and is available under the name MAXAZYM® CL-2000 (IBIS, N.V., Rijswijk, The Netherlands). The enzymatic activities of this product are summarized in Table 1, below.

Table 1: Enzymatic activities of Cellulase ABG-7

30	ACTIVITY	MEASURED PRODUCT	SPECIFIC ACTIVITY μmoles/min.Mg protein
35	CMC-ase	glucose equivalent	0.79
	Avicelase	glucose equivalents	0.11
	Endoxylanase	xylose equivalents	16.0
	Exoxyylanase	p-nitrophenol	0.48
	Exoarabinase	p-nitrophenol	0.51
	Acetyl esterase	p-nitrophenol	0.16
	Polygalacturonase	galactose equivalents	3.56
	Glucuronidase	p-nitrophenol	0.003
40	α-Amylase	glucose	0.25

- 12 -

The results of the silage experiment are presented in Table 2, below.

Table 2: Mean (N=3) composition of grass, control silage and enzyme-treated silage after 5 incubation with Cellulase ABG-7

			SILAGE		
			GRASS	CONTROL	CELLULASE ABG-7
10	Dry matter (DM)	(g/kg)	273	269	259 *
	Ash	(g/kg DM)	124	124	136 *
15	Lactic acid	(g/kg DM)	-	45.5	59.4 *
	Ethanol	(g/kg DM)	-	7.33	19.1 *
	Acetic acid	(g/kg DM)	-	46.9	82.8 *
	Crude fiber	(g/kg DM)	258	262	194 *
20	Neutral Detergent Fiber	(g/kg DM)	523	446	351 *
	Acid Detergent Fiber	(g/kg DM)	279	281	204 *
	Acid Detergent Lignin	(g/kg DM)	17.5	15.9	18.5 *
25	Weight loss	(g/kg DM)	-	49.9	76.3 *
	NH ₃ -N/total N	(wt% N-total)	-	26	20 *
	pH	-	-	5.42	4.72

* statistically different from the control.
P < 0.05 (Student T-test)

30 Cellulase ABG-7 hydrolysed a large proportion of cell wall constituents to fermentable carbohydrates. This resulted in increased silage quality as characterized by the liberation of water soluble carbohydrates. This is evidenced 35 by the increased lactic acid concentration and lower crude fiber content, lower Neutral Detergent Fibre content, lower Acid Detergent Fiber content, as well as lower pH and ammonia values in comparison to the control.

40 Example 3

Perennial English rye-grass was prepared as described in Example 2, above, and ensiled in 1 liter laboratory silos and stored for 3 months at ambient temperature before analysis.

- 13 -

The enzyme Cellulase ABG-7 (see Table 1, above) was added at concentrations of 0.005 g of protein (80 units endo-xylanase activity) per kg fresh weight grass; 0.025 g of protein (400 units endo-xylanase activity) per kg fresh weight grass; 0.050 g of protein (800 units endo-xylanase activity) per kg fresh weight grass; 0.075 g of protein (1200 units endo-xylanase activity) per kg of fresh weight grass (all protein content determined using the method as described by Bradford, M.M. (supra)).

10 The results are presented in Table 3.

Table 3 : Mean (N=3) composition of grass and silages.

	GRASS	CONTROL SILAGE	SILAGE			
			0.005	0.025	0.050	0.075
Dry matter (DM)	(g/kg)	233	223	223	221	*
Ash	(g/kg DM)	111	117	102	116	126
Lactic acid	(g/kg DM)	-	5.73	4.22*	4.44*	4.97*
Ethanol	(g/kg DM)	-	33.7	26.5 *	20.0 *	32.6
Acetic acid	(g/kg DM)	-	249	252	247	242
Crude fibre	(g/kg DM)	256	429	430	427	413
NDF	(g/kg DM)	505	428	266	261	257
ADF	(g/kg DM)	275	268	20.4	21.8	20.8
ADL	(g/kg DM)	19.7	20.8	39.9	26.9 *	25.8 *
Weight loss	(g/kg DM)	-	39.9	26.9 *	25.8 *	34.7
NH ₃ -N/total N (wt % N-total)	-	-	16	14 *	11 *	14 *
pH	-	-	4.25	4.09*	3.93*	4.10*

* statistically different from the control. P < 0.05 (Student T-test)

- 15 -

Example 4

Perennial English rye-grass was prepared as described in Example 2, above, and ensiled in 1 liter laboratory silos and stored for 3 months at ambient temperature before analysis.

In this experiment, a purified endoxylanase from Aspergillus tubingensis (specific activity 5000 units endoxylanase activity per kg fodder (Bradford protein determination method, supra); see Example 1) was combined with the Cellulase ABG-7 preparation to increase the endoxylanase activity in the preparation and added to the grass. The endoxylanase concentration in this combined enzyme product added to the grass was 0.050 g of protein (250,000 units endo-xylanase activity) per kg (fresh weight) grass and the Cellulase ABG-7 concentration added was 0.200 g of protein (3200 units endo-xylanase activity) per kg (fresh weight) grass (protein contents determined using the method as described by Bradford, M.M. (supra)).

The results are presented in Table 4.

20

Table 4 : Mean (N=3) composition of grass and silages.

25

SILAGE

30

		GRASS	CONTROL	CELLULASE ABG-7	CELLULASE ENDOXY- LANASE	
	Dry matter (DM)	(g/kg)	273	269	259	*
	Ash	(g/kg DM)	124	124	136	*
	Lactic acid	(g/kg DM)	-	45.5	59.4	*
35	Ethanol	(g/kg DM)	-	7.33	19.1	*
	Acetic acid	(g/kg DM)	-	46.9	82.8	*
	Crude fibre	(g/kg DM)	258	262	194	*
	NDF	(g/kg DM)	523	446	351	*
	ADF	(g/kg DM)	279	281	204	*
40	ADL	(g/kg DM)	17.5	15.9	18.5	*
	Weight loss	(g/kg DM)	-	49.9	76.3	*
	NH ₃ -N/total N (wt % N-total)		-	26	20	*
	pH		-	5.42	4.72*	4.39*

45

* statistically different from the control. P < 0.05

- 16 -

surprisingly, the addition of endo-xylanase to the Cellulase ABG-7 preparation resulted in a much higher concentration of lactic acid in the ensiled grass and subsequently a much lower pH.

5 Moreover the fermentation of ethanol and acetic acid is suppressed as well as the weight loss due to the formation of CO₂ and volatile fatty acids.

Example 5

10 Perennial English rye-grass was prepared as described in Example 2, above, and ensiled in 1 liter laboratory silos and stored for 3 months at ambient temperature before analysis.

15 Purified endoxylanase was added to the grass in concentrations ranging of 0.010 g, 0.050 g and 0.200 g of protein per kg fresh weight fodder (Bradford protein determination method, supra).

The results are presented in Table 5.

Table 5 : Mean (N=3) composition of grass and silages.

5	GRASS	CONTROL SILAGE	SILAGE		
			ENDO ₁ (units/kg grass)	10 ⁶	
				50,000	250,000
10	Dry matter (DM)	(g/kg DM)	273	269	267
	Ash	(g/kg DM)	124	126	127
	Lactic acid	(g/kg DM)	-	45.5	50.0
	Ethanol	(g/kg DM)	-	7.33	6.54
15	Acetic acid	(g/kg DM)	-	46.9	48.2
	Crude fibre	(g/kg DM)	258	262	263
	NDF	(g/kg DM)	523	446	438
	ADF	(g/kg DM)	279	281	283
	ADL	(g/kg DM)	17.5	15.9	16.8
20	Weight loss	(g/kg DM)	-	49.9	51.4
	NH ₃ -N/total N (wt % N-total)	-	-	26	27
	pH	-	-	5.42	5.23
					4.70*

* statistically different from the control. P < 0.05 (student T-test)

- 18 -

The results presented in Table 5 clearly demonstrate the efficacy of the supplementation of endo-xylanase activity in the production of an improved silage. The data illustrate a substantial increase of lactic acid concentration in the ensiled grass with increasing amounts of supplemented endo-xylanase activity. A slight increase of acetic acid content was observed. This in addition to the increased lactic acid content account for the lower pH and thus a better preservation of the silage. No significant increase in ethanol production (from undesirable yeast fermentation) was observed. Moreover, the data show a significant decrease of cell wall biopolymer parameters (lower crude fiber content, lower Neutral Detergent Fibre content and lower Acid Detergent Fiber content in comparison to the control), resulting in an increase in fermentable sugars available for lactic acid bacteria and for an animal consuming an endo-xylanase treated silage. The pre-digestion of the cell wall material also results in the enhanced digestibility of the ensiled fodder.

- 19 -

Claims:

1. An endo-xylanase-containing silage composition containing more than 50,000 units endo-xylanase activity per 5 kg (fresh weight) fodder.
2. The silage composition of claim 1, further characterized in that the endo-xylanase is obtained from a filamentous fungus.
3. The silage composition of claim 2, further characterized in that the endo-xylanase is obtained from a filamentous fungus selected from the genera consisting of Aspergillus, Disporotrichum, Penicillium, Neurospora, 10 Fusarium and Trichoderma.
4. The silage composition of claim 3, further characterized in that the endo-xylanase is obtained from a filamentous fungus selected from the species consisting of 20 Aspergillus niger, Aspergillus awamori, Aspergillus aculeatus, Aspergillus tubigensis, Disporotrichum dimorphosporum and Trichoderma reesei.
5. The silage composition of claim 1, further 25 characterized in that the endo-xylanase has a pH optimum in the range of pH 3.5 to pH 6.0.
6. The silage composition of claim 1, further characterized in that it further contains an inoculum of a 30 lactic acid bacteria.
7. The silage composition of claim 6, further characterized in that the lactic acid bacteria is selected from the group consisting of Lactobacillus plantarum, 35 Lactobacillus acidophilus, Lactobacillus brevis, Lactobacillus bulgaricus, Lactobacillus fermentum and Lactobacillus lactis.

- 20 -

8. A silage composition according to any one of claims 1-7, further characterized in that the endo-xylanase is supplemented to the fodder in a form substantially free of 5 other enzymes.

9. A method of preserving silage characterized in that the silage is supplemented with an amount of endo-xylanase activity effective in increasing the amount of water soluble 10 carbohydrates present in the silage for use in the production of lactic acid and acetic acid by lactic acid bacteria.

10. A method for improving the in vivo digestibility of 15 silage in animals wherein the animal is fed a diet containing silage which has been supplemented with an amount of endo-xylanase activity effective in at least partially degrading plant cell walls.

11. A method for the improvement of the nutritional value 20 of silage by supplementing the silage with an amount of endo-xylanase activity effective in releasing water soluble carbohydrates from the ensiled plant cell material for utilization by the animal ingesting the silage.

25 12. Use of an endo-xylanase in the preparation of silage.

13. A composition comprising at least 50 wt. % endo- 30 xylanase for use in the treatment of silage.

14. A silage treated with more than 50,000 units endo- xylanase/kg (fresh weight) fodder.

15. An animal feedstuff composition comprising the silage 35 of claim 14.

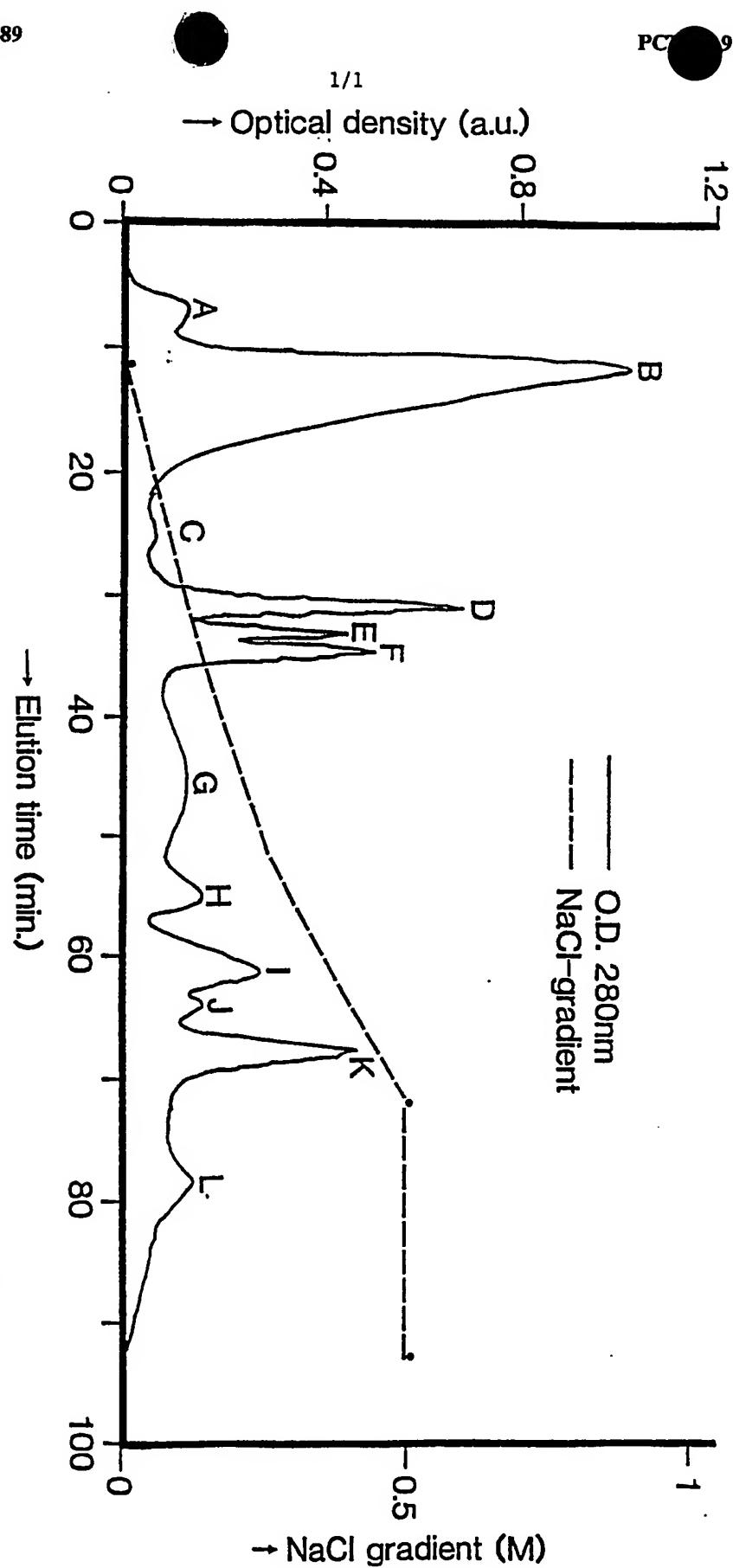


Figure 1

INTERNATIONAL SEARCH REPORT

International Application No

PCT/NL 91/00136

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) ⁶		
According to International Patent Classification (IPC) or to both National Classification and IPC Int.C1. 5 A23K3/03 ; C12N9/24 ; A23K1/165		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
Int.C1. 5	A23K ; C12N	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸		
III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹		
Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
X	CHEMICAL ABSTRACTS, vol. 87, no. 22, November 28, 1977, Columbus, Ohio, US; abstract no. 182914, SILVERS V. S. ET AL 'Use of hydrolytic enzyme preparations during ensilage of corn cobs' page 484 ;	9,12
A	see abstract ---	1-8, 10-11, 13-15 -/-
<p>⁶ Special categories of cited documents : 10</p> <p>⁷ "A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>⁸ "E" earlier document but published on or after the international filing date</p> <p>⁹ "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>¹⁰ "O" document referring to an oral disclosure, use, exhibition or other means</p> <p>¹¹ "P" document published prior to the international filing date but later than the priority date claimed</p>		<p>¹² "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>¹³ "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>¹⁴ "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>¹⁵ "A" document member of the same patent family</p>
IV. CERTIFICATION		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
2 17 SEPTEMBER 1991	24.09.91	
International Searching Authority EUROPEAN PATENT OFFICE	Signature of Authorized Officer LE CORNEC N.D.R.	

III. DOCUMENTS CONSIDERED TO BE RELEVANT		(CONTINUED FROM THE SECOND SHEET)
Category	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	CHEMICAL ABSTRACTS, vol. 94, no. 11, March 16, 1981, Columbus, Ohio, US; abstract no. 82520X, BODGAN S. D. ET AL 'Use of enzymic preparations for the ensilage of wheat straw.' page 591 ;	9,12
A	see abstract ---	1-8, 10-11, 13-15
X	CHEMICAL ABSTRACTS, vol. 93, no. 3, July 21, 1980, Columbus, Ohio, US; abstract no. 24758Z, TASHPULATOV,ZH: 'Microbial processes in the ensiling of guza-paya using cellulolytic enzymes.' page 567 ;	9,11-12
A	see abstract ---	1-8,10, 13-15
A	O. JORGENSEN ET AL. , In: 'Enzyme systems for lignocellulose degradation, Ed. M.P. Coughlan.' 1989 , ELSEVIER APPLIED SCIENCE , LONDON, GB. see page 347 - page 355 ---	1-15
A	CHEMICAL ABSTRACTS, vol. 110, no. 25, June 25, 1989, Columbus, Ohio, US; abstract no. 230420F, NAKASHIMA Y. ET AL: 'Rumen degradation of straw.6. Effect of polysaccharidase enzymes on degradation characteristics of ensiled rice straw.' page 535 ; see abstract ---	1-15
A	WO,A,8 910 066 (CULTOROY) see claims; example 1 ----	1-15

ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.

NL 9100136
SA 49974

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

17/09/91

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
<hr/>				
WO-A-8910066	02-11-89	AU-A- EP-A-	3449389 0412094	24-11-89 13-02-91